

Original Research Article

## Toxicity of Crude Oil and Diesel Fuel to Some Gram Positive and Gram Negative Bacteria

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### Abstract

Oil spillage poses a serious threat to the biosphere. In this study, toxicity of crude oil and diesel fuel to some Gram positive and Gram negative bacteria was investigated. A total of twelve isolates belonging to the bacterial genera *Erwinia*, *Shigella*, *Escherichia*, *Morganella*, *Micrococcus*, *Staphylococcus*, *Proteus*, *Serratia*, *Lactobacillus*, *Klebsiella*, *Bacillus* and *Pseudomonas* were isolated from pristine soil samples and characterized. The toxicity of these organisms was assessed by their ability or otherwise to grow in tryptic soy broth to which different concentrations (1%, 5%, 10%, 15%, and 20%) of the toxicants (crude oil and diesel fuel) was respectively added. The bacterial counts of the samples to which crude oil was added ranged from  $65.97 \pm 29.78$  to  $79.92 \pm 22.94$  and varied significantly ( $p < 0.05$ ) among concentrations. The bacterial counts of the samples to which diesel fuel was added ranged from  $49.53 \pm 30.11$  to  $79.36 \pm 22.45$  and varied significantly ( $p < 0.05$ ) among concentrations. Diesel fuel was found to be more toxic than the crude oil on the bacterial species used in this study. The toxicity of crude oil and diesel fuel increased with increase in concentrations. However, *Erwinia cacticida* and *Klebsiella pneumoniae* subsp. *ozaenae* showed good tolerance to both crude oil and diesel fuel even at high concentrations of 20%. These organisms could play a useful role in the bioremediation of oil polluted environments.

### Article Info

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### Keywords

Crude oil  
Diesel fuel  
Oil spillage  
Toxicity

### Introduction

Petroleum and their derivative are a major source of energy in the world today. Petroleum hydrocarbons provide over 50% of the energy used all over the world. In addition, petroleum is the principal source of lubricants, solvents and a variety of chemical feedstock for synthesis of plastics, fibres, detergents, pharmaceuticals and cosmetics. The large scale of operation necessitated by the above demands cited, make the petroleum industry a potential source of air,

water and soil pollutants. Generally, petroleum hydrocarbons vary from simple aliphatic and aromatic compounds to complex, multi-ring structure of high molecular weight. They also contain a wide range of substances that contain sulfur, nitrogen, oxygen and other elements. According to Narve (2002), "crude oils are a continuum of tens of thousands of different hydrocarbon molecules". Crude oils consist, primarily, straight chains hydrocarbons (alkanes) cycloalkanes and various aromatics hydrocarbons. Diesel is a petroleum-based fuel for diesel engines. It is a thick oily fuel that is

obtained from the distillation of petroleum. In addition, diesel fuel is a mixture of complex molecules called hydrocarbons and it “contains low molecular weight compounds that are usually more toxic than long chained hydrocarbons, because long-chained ones are less soluble and less bioavailable” (Dorn and Salanitro, 2000). Its low molecular weight nature makes it lighter and “light oils contain a relatively high proportion of saturated hydrocarbons hence these can be more toxic than heavy oils” (Dorn et.al., 1998) “Petroleum-derived diesel is composed of about 75 percent saturated hydrocarbons (primarily paraffins including n, iso, and cycloparaffins), and 25 per cent aromatic hydrocarbons (including naphthalenes and alkylbenzenes)” (Atlas and Bartha, 1995). Among the aromatic fraction of petroleum hydrocarbon, polycyclic aromatic hydrocarbons (PAHs) are unusual class of petroleum hydrocarbons because of the complexity of the assemblages in which they occur and their pyrogenic nature. PAHs are recalcitrant pollutants, which have toxic, mutagenic and carcinogenic properties and “are listed among the priority pollutants by the U.S. Environmental Protection Agency” (USEPA, 2012).

Crude oil and diesel fuel are a major source environmental pollution. The pollution occurs from various sources including human error, equipment failure, deliberate vandalism, or disasters (Anderson and LaBelle, 2000); and increased demand for diesel fuel for some vehicles and generators resulted in larger quantities of this product to be transported over long distances. Microorganisms perform a crucial function on nutritional chains which are relevant component of the biological balance. Thus microorganisms play an important role in ecological systems. There are several studies on the biodegradability of crude oil and diesel fuel and other petrochemical compounds by microorganisms in the soil, estuaries and marine environment (Tazeena et.al., 2013; USCOTA, 1991; Okerentugba and Ezeronye, 2003). These petrochemicals contain one or more toxic compounds, some of which are degradable by some microorganisms (particularly hydrocarbon degraders) while some remain in the soil, and may have deleterious effect on other microorganisms (non-hydrocarbon degraders). Microorganisms have evolved ability to regulate some aspect of their life behavior in response to any change in their environment.

Studies have shown that large numbers of indigenous microorganisms inhabiting polluted environment are

those capable of utilizing one or more components of the pollutant probably as source of carbon and/ or energy (Okerentugba and Ezeronye, 2003). However, few studies have been documented in the literatures on tolerance of indigenous microorganisms in pristine soil to the toxicity of crude oil and diesel fuel. This study attempts to fill this gap, by isolating and identifying those microbes with some degree of tolerance to toxicity of crude oil and diesel fuel.

## Materials and methods

### Sampling locations

In this study, eight (8) locations were surveyed and identified. All locations were situated around Export Processing Zone (EPZ) and its environs, and University of Calabar and its environs in Calabar, Cross River State- Nigeria.

### Sample collection

Approximately 500 g of surface and subsurface pristine soil samples were collected into sterile plastic covered plates using soil auger. Collected samples were transferred under sterile condition to the microbiology laboratory and stored at 4°C until needed for analysis. Diesel fuel was obtained from Oryx depot EPZ, Calabar, Cross River State. Bonny light crude oil sample was obtained from Exxon- Mobile Nig. Ltd. Qua Iboe terminal, Ibeno, Akwa Ibom State.

### Isolation of total heterotrophic bacteria

Ten grams (10g) of soil sample was weighed into 90 ml distilled water in 200 ml holding capacity bottle and capped. The soil suspension was agitated vigorously to dislodge bacteria from soil particles and then allowed to stand for few minutes. Series of ten-fold dilutions were prepared from the initial dilution. 0.1 ml of  $10^{-3}$  to  $10^{-5}$  dilutions was spread plated onto nutrient agar supplemented with antifungal agents (50µg/ml of nystatin and 75µg/ml of cycloheximide). Triplicate plating was carried out; the plates were sealed with petriseal and incubated at average ambient temperature of 28°C for 48 hrs.

### Identification of bacterial isolates

The bacterial colonies were identified by Gram staining and biochemical tests according to methods recorded in

Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1997).

### Tolerance of microorganisms to different concentrations of toxicants (crude oil and diesel fuel)

A method described by Nseabasi and Antai (2012) was adopted and modified. Tolerance to crude oil and diesel fuel at different concentrations were assessed by the ability of organisms to grow in Tryptic soy broth into which crude oil and diesel fuel was incorporated at different concentrations of 1%, 5%, 10%, 15%, and 20%. These Tryptic soy broth (TSB) tubes (9ml each) containing crude oil and diesel fuel at different concentrations were inoculated with one (1.0) ml of 24 hrs old broth culture of some selected bacteria species and were agitated vigorously to mix the broth culture with the toxicant. The inoculated broth tubes were incubated at average ambient temperature of 28°C for 24 hrs. After twenty-four (24) hours of incubation, 0.1ml of each tube was spread plated on TSA agar plate and incubated at average ambient temperature of 28°C for 24 hrs. The same process was applied to Tryptic soy broth (TSB) tubes with no inclusion of crude oil and diesel fuel to serve as control for the basis of comparison. After the incubation period, tolerance range of crude oil and diesel fuel at different concentrations on some selected bacteria species were obtained on the basis of their growth (number). The number of colonies were counted and expressed in colonies forming unit (CFU/ml). The measuring unit of the toxicants was in millilitre (ml).

### Percent-log survival test

Williamson and Johnson (1981) formula was adopted to calculate percent-log survival of bacteria isolate. This was done by obtaining the log of count in each toxicant concentration (Log C) respectively and dividing by log of count in the zero (control) toxicant concentration (Log c) and multiplying by 100.

$$\% \text{ log survival} = (\text{Log C} / \text{Log c}) \times 100$$

### Statistical analysis

Analytical software (Minitab version 17) was used in analyzing the data obtain from this study. Means of crude oil and diesel fuel tolerant bacteria isolates were compared using one way Analysis of Variance (ANOVA) and Hsu simultaneous (95% CIs) was used to test for significant difference among means. Hsu Multiple Comparisons with the Best (Hsu MCB) was used to identify factor levels that are the best, non-significantly different from the best, and those that are significantly different from the best. Dunnett Simultaneous (95% CIs) was used to test for difference between each treatment group and the control group. All statistical testing was performed at 95% confidence interval.

### Results

A total of 107 bacterial species were isolated and characterised from the pristine soil samples. Twelve (12) out of the 107 isolates were randomly selected and used for the toxicants' tolerance studies. They include *Erwinia cacticida*, *Shigella sonnei*, *Escherichia coli*, *Morganella marganii*, *Micrococcus luteus*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, *Serratia liquefaciens*, *Lactobacillus delbrueckii*, *Klebsiella pneumoniae* subsp. *ozaenae*, *Bacillus* sp. and *Pseudomonas* sp. The bacterial counts of the samples to which crude oil was added ranged from  $65.97 \pm 29.78$  and  $79.92 \pm 22.94$  and varied significantly ( $p < 0.05$ ) among concentrations. The bacterial counts of the samples to which diesel fuel was added ranged from  $49.53 \pm 30.11$  and  $79.36 \pm 22.45$  and varied significantly ( $p < 0.05$ ) among concentrations. Comparatively, the mean values of the samples to which crude oil was added were significantly ( $p < 0.05$ ) higher from those of the samples to which diesel fuel was added (Table 1).

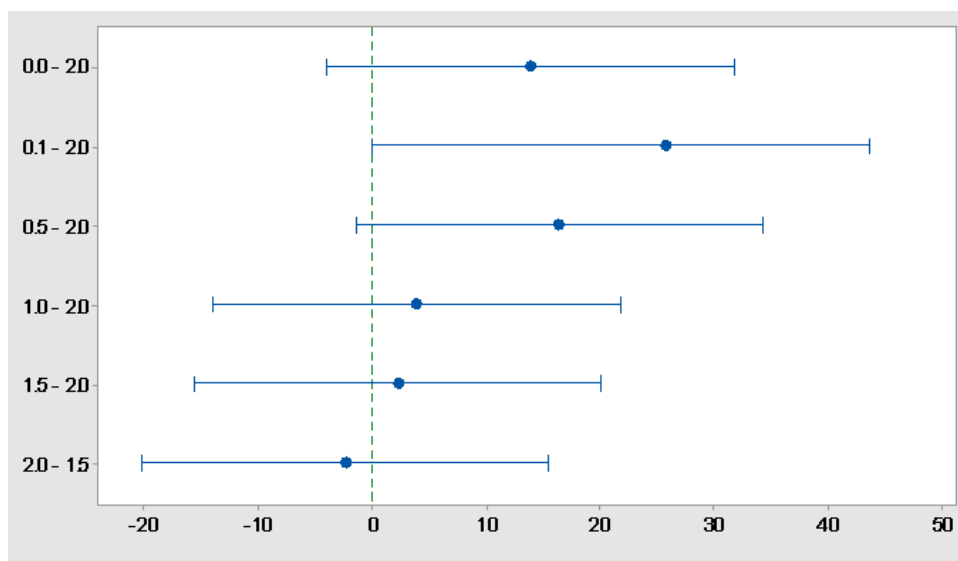
**Table 1.** Inter-concentration and toxicant comparison of bacteria isolates from pristine soil.

Toxicant	Concentration						p-value
	0.0	0.1	0.5	1.0	1.5	2.0	
Crude oil	79.92 ± 22.94 <sup>a</sup>	91.83 ± 48.32 <sup>b</sup>	82.44 ± 33.90 <sup>c</sup>	69.89 ± 31.41 <sup>d</sup>	68.31 ± 30.43 <sup>e</sup>	65.97 ± 29.78 <sup>f</sup>	0.008 <sup>S</sup>
Diesel oil	79.36 ± 22.45 <sup>a</sup>	93.56 ± 59.45 <sup>b</sup>	80.92 ± 52.45 <sup>c</sup>	66.03 ± 42.41 <sup>d</sup>	56.03 ± 33.35 <sup>e</sup>	49.53 ± 30.11 <sup>f</sup>	0.000 <sup>*S</sup>

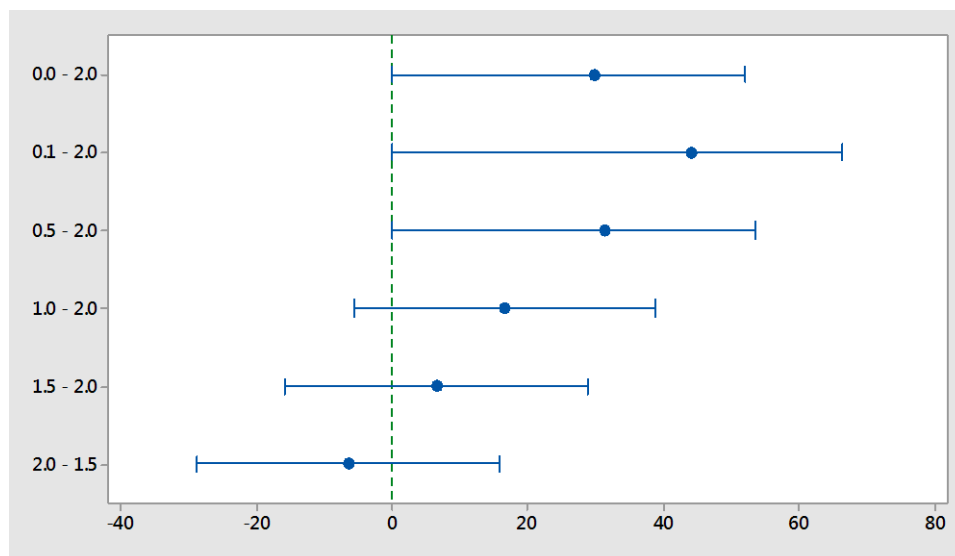
S = Significant, \* = More significant, Mean ± SD, mean in row with the superscript letter (a,b,c,d,e,f) are significantly different from each other (Hsu simultaneous 95% CIs).

Figs. 1 and 2 show the level mean - smallest of other level means for growth for crude oil and diesel fuel respectively. There was a statistically significant difference between the corresponding concentration means for both the crude oil and diesel oil (Hsu simultaneous 95% CIs). The means of concentration 0% (control), 1%, 5%, 10%, and 15% and were compared to the concentration 20% mean because it is the lowest (lowest mean was chosen to be the best concentration that shows more toxic effect to bacteria growth). Concentration 5%, 10% and 15% were significantly better for crude oil while only concentration 10%, 15%

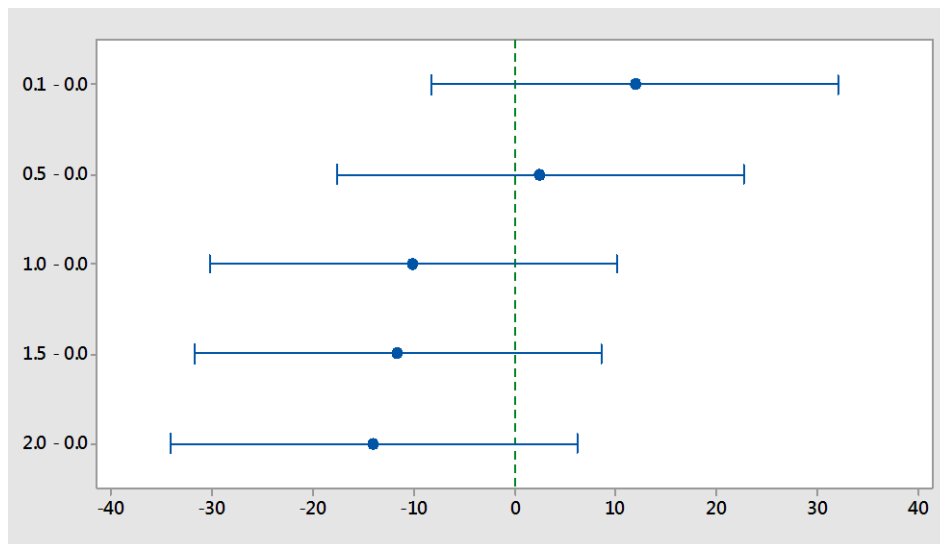
were significantly best for diesel fuel. The confidence intervals for differences between the mean of each concentration and the mean of a control group for crude oil and diesel fuel are presented in Figs. 3 and 4 respectively. There was no significant difference between the two means under comparison in both crude oil and diesel fuel (Dunnett Simultaneous 95% CIs). Figs. 5 and 6 presented main effects plot of crude oil at different concentration and growth response of each organism and main effects plot of diesel fuel at different concentration and growth response of each organism respectively.



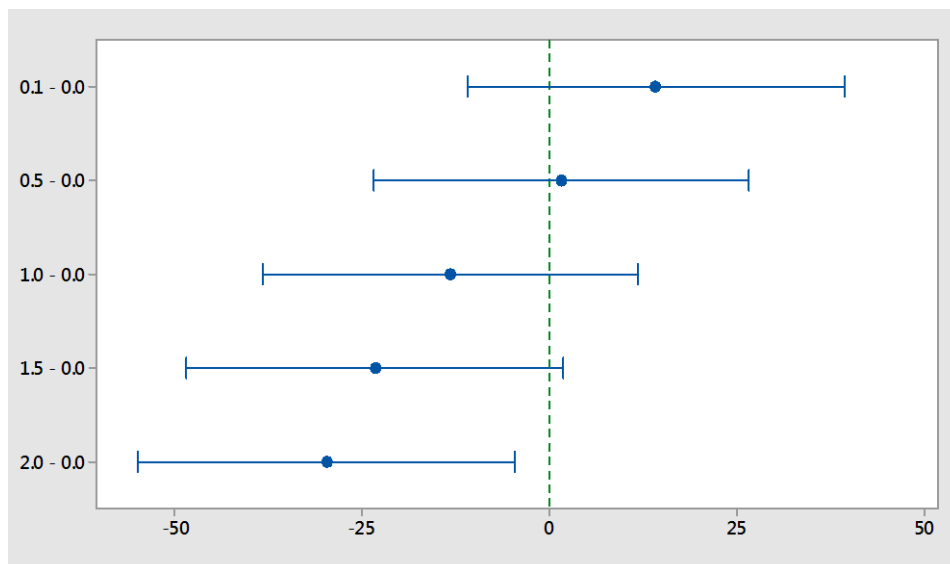
**Fig. 1:** Level mean - smallest of other level means for growth (crude oil) (Hsu simultaneous 95% CIs).  
\*If an interval has zero as an endpoint, the corresponding means are significantly different.



**Fig. 2:** Level mean - smallest of other level means for growth (diesel fuel) (Hsu simultaneous 95% CIs)  
\*If an interval has zero as an endpoint, the corresponding means are significantly different.



**Fig. 3:** Level mean - control mean for growth for crude oil (Dunnett Simultaneous 95% CIs).\*If an interval does not contain zero, the corresponding mean is significantly different from the control mean.



**Fig. 4:** Level mean - control mean for growth for diesel fuel (Dunnett simultaneous 95% CIs).\*If an interval does not contain zero, the corresponding mean is significantly different from the control mean.

Generally, the main effect of crude oil and diesel fuel to bacteria increased with increase in concentration. The bacteria growth decreased with increase in concentration of diesel fuel, while at concentration of 15% for crude oil; there was no significant difference in bacteria growth compare to concentration of 20%. The growth responses of bacteria isolated from pristine soil were difference when exposed to crude oil and diesel fuel. Statistically *Erwinia cacticida*, *Klebsiella pneumoniae* subsp. *ozaenae*, *Serratia liquefaciens*, and *Pseudomonas* sp. growth were above the average. The growth responses were in order *Klebsiella pneumoniae* subsp. *ozaenae* > *Erwinia cacticida* > *Serratia liquefaciens* >

*Pseudomonas* sp. for crude oil and *Erwinia cacticida* > *Klebsiella pneumoniae* subsp. *ozaenae* > *Pseudomonas* sp. > *Serratia liquefaciens* for diesel oil (Figs. 7 and 8). Crude oil and log survival percentage of Gram negative organism at different concentration of diesel fuel. Gram negative bacteria, *Erwinia cacticida*, *Klebsiella pneumoniae* subsp. *ozaenae*, *Serratia liquefaciens*, and *Pseudomonas* sp. survived crude oil at all concentrations and the survival rate was the same at concentration of 10% -20%. In diesel fuel, Gram negative bacteria, *Erwinia cacticida*, *Klebsiella pneumoniae* subsp. *ozaenae* survived the concentration at 1%-10% while *Pseudomonas* sp. survival rate was the same at all the

concentrations. Log survival percentage of Gram positive organism at different concentration of crude oil and log survival percentage of Gram positive organism at different concentration of diesel fuel are presented in Figs. 9 and 10 respectively.

In crude oil, Gram positive bacteria, *Bacillus* sp. survival

rate was the same at all concentrations, *Micrococcus luteus* and *Staphylococcus saprophyticus* survived the concentration at 1% – 10% and 1% – 5% respectively. While in diesel fuel, *Bacillus* sp. survival rate also was the same at all concentrations, *Micrococcus luteus* and *Staphylococcus saprophyticus* survived the concentration at 1% – 15% and 1% respectively.

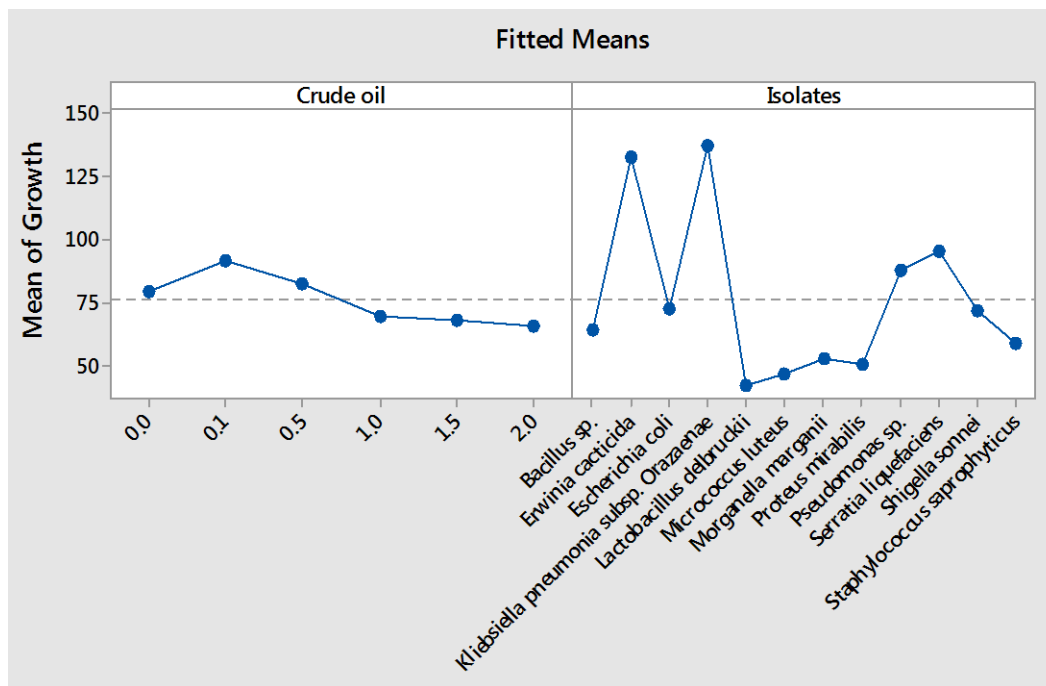


Fig. 5: Main effects plot of crude oil at different concentration and growth response of each organism.

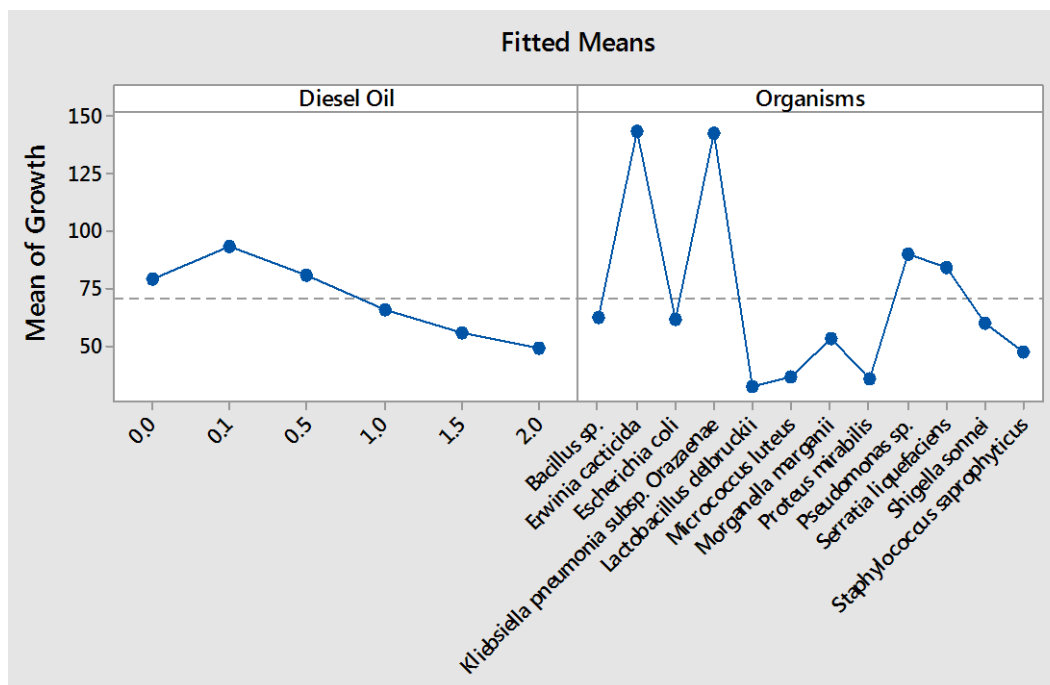


Fig. 6: Main Effects Plot of diesel fuel at different concentration and growth response of each organism.

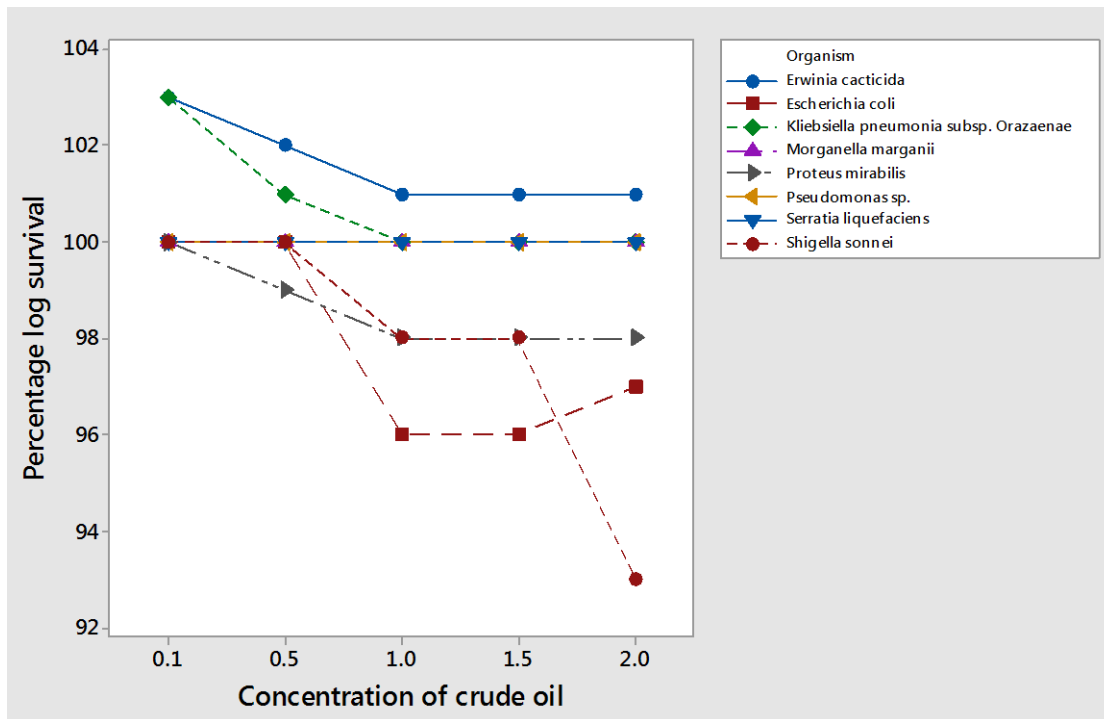


Fig. 7: Log survival percentage of Gram negative organisms at different concentration of crude oil.

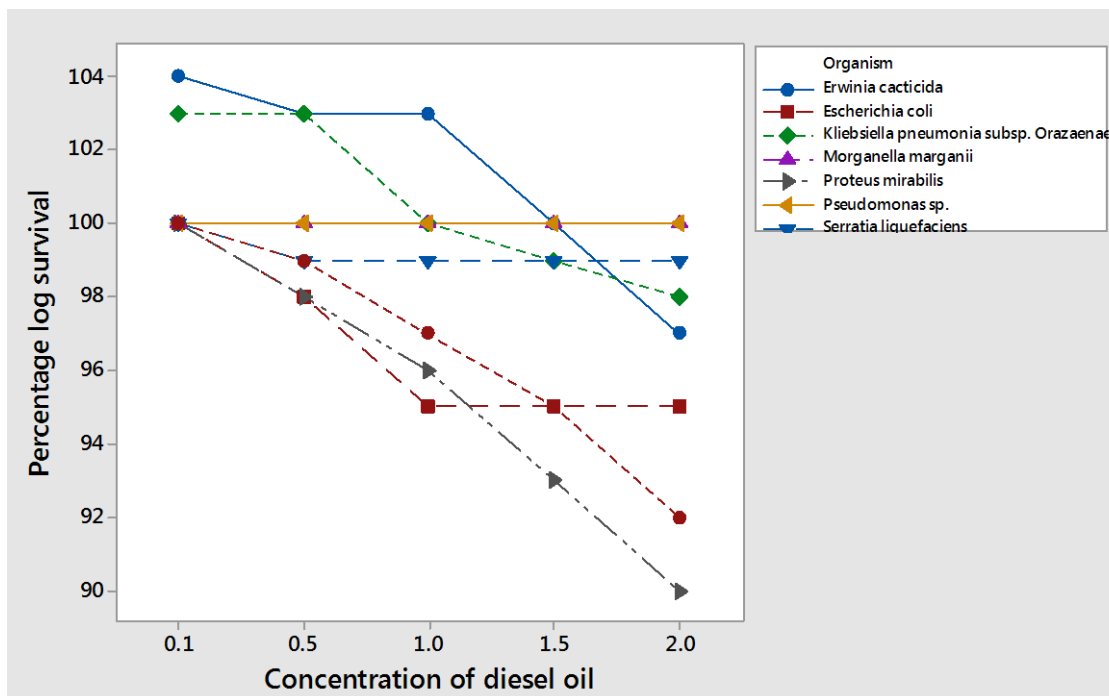


Fig. 8: Log survival percentage of Gram negative organisms at different concentration of diesel fuel.

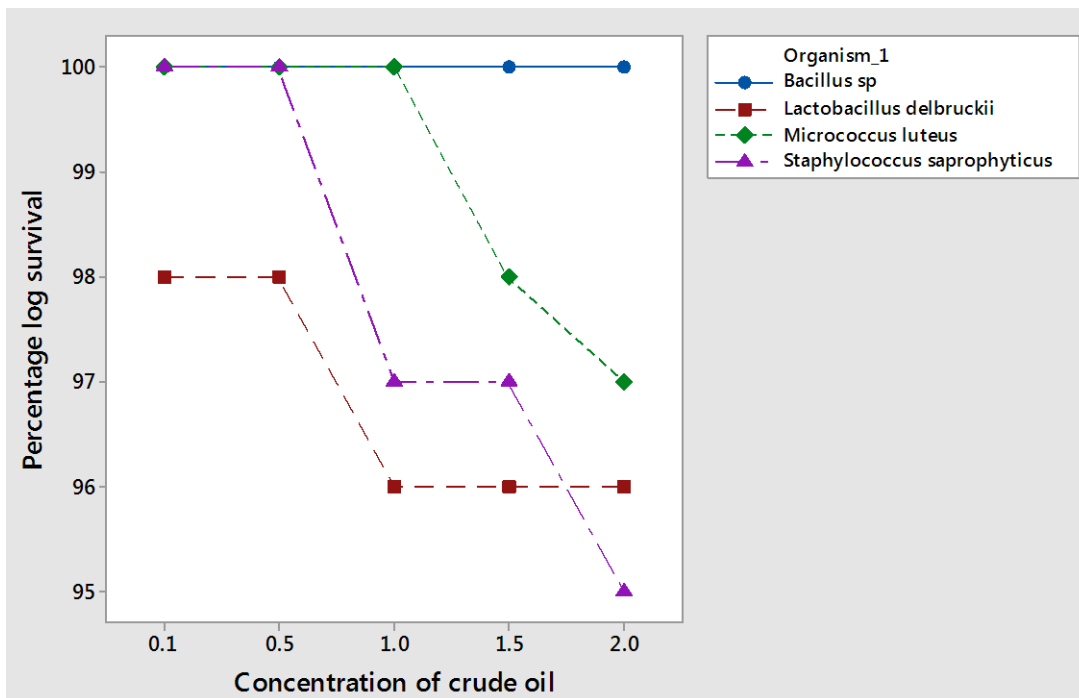


Fig. 9: Log survival percentage of Gram positive organisms at different concentration of crude oil.

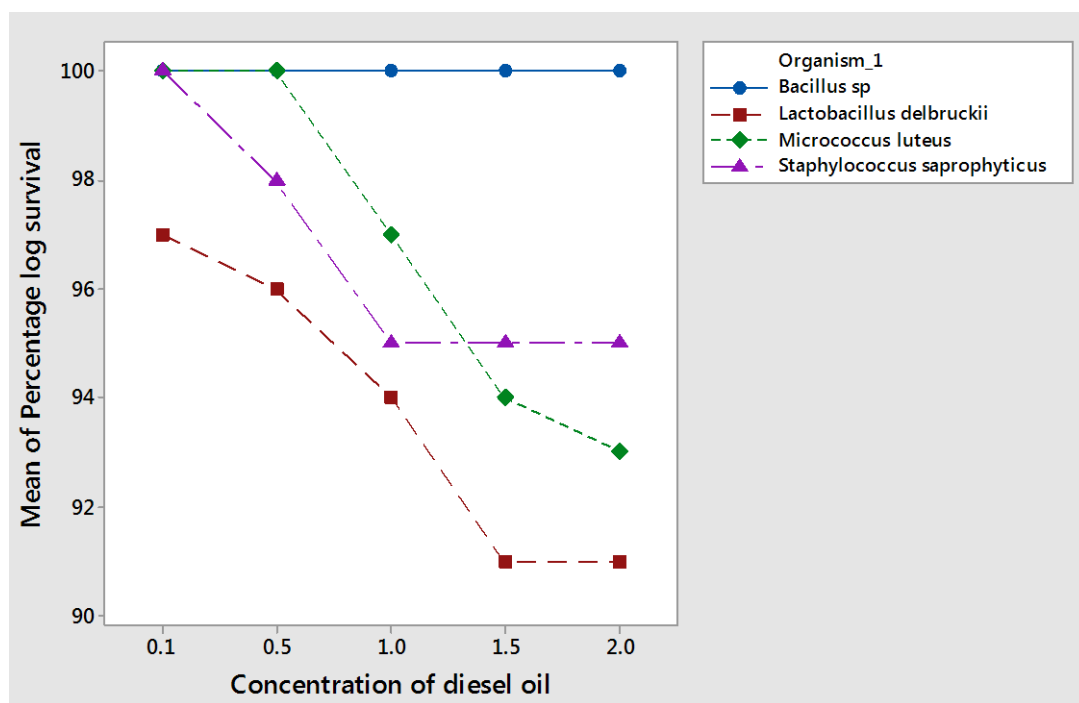


Fig. 10: Log survival percentage of Gram positive organisms at different concentrations of diesel oil.

## Discussion

The results in this study revealed that crude oil and diesel fuel have a great impact on the isolated bacteria. This impact was obvious from the bacterial population count which varied between isolated bacteria at different

concentrations of crude oil and diesel fuel. Diesel fuel is known to contain light fraction of hydrocarbons and hence more soluble. Hydrocarbons possessing more than 11 carbon atoms (C<sub>12</sub> – C<sub>42</sub>) are less harmful to soil microorganisms than light fraction hydrocarbons (Wyszkowska and Kucharski, 2002). This property



makes diesel fuel more toxic, contrary to crude oil with heavier fraction of hydrocarbons. In large quantity, crude oil and diesel fuel are toxic to soil microorganisms. However, crude oil and diesel fuel were found to have both positive and negative effect on growth of bacteria isolates studied. Although, the growth of all the bacteria isolates, *Erwinia cacticida*, *Shigella sonnei*, *Escherichia coli*, *Morganella morganii*, *Micrococcus luteus*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, *Serratia liquefaciens*, *Lactobacillus delbrueckii*, *Klebsiella pneumoniae* subsp. *ozaenae*, *Bacillus* sp. and *Pseudomonas* sp. decreased with increasing crude oil and diesel fuel concentration but the growth response of *Erwinia cacticida* and *Klebsiella pneumoniae* subsp. *Ozaenae* appeared to be enhanced by the crude oil and diesel fuel and decreased with increase in concentration of crude oil and diesel fuel.

Perhaps, these organisms, *Erwinia cacticida* and *Klebsiella pneumoniae* subsp. *Ozaenae* possess a unique physiological property to metabolise oil as energy source despite the polycyclic aromatic hydrocarbons (PAHs) in the oil. The growth of other organisms, *Shigella sonnei*, *Escherichia coli*, *Morganella morganii*, *Micrococcus luteus*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, *Serratia liquefaciens*, *Lactobacillus delbrueckii*, *Bacillus* sp. and *Pseudomonas* sp. was greatly affected by the mutagenic characteristics (Kanaly and Harayama, 2000) of polycyclic aromatic hydrocarbons (PAHs).

Gram negative organisms, *Erwinia cacticida*, *Shigella sonnei*, *Escherichia coli*, *Morganella morganii*, *Proteus mirabilis*, *Serratia liquefaciens*, *Klebsiella pneumoniae* subsp. *Ozaenae*, and *Pseudomonas* sp. showed higher tolerance to toxicity of crude oil and diesel fuel compared to Gram positive isolates, *Micrococcus luteus*, *Staphylococcus saprophyticus*, *Lactobacillus delbrueckii* and *Bacillus* sp. This may be due to complexity of Gram negative cell wall (<http://micro.digitalproteus.com/morphology2.php>).

Generally, organic solvents are toxic to bacteria cells even at very low concentration (0.1%) (Stancu, 2014). Gram negative organisms, *Erwinia cacticida*, *Klebsiella pneumoniae* subsp. *ozaenae*, and *Pseudomonas* sp. showed good tolerance to 20% concentrations of crude oil, but in diesel fuel, *Erwinia cacticida* and *Klebsiella pneumoniae* subsp. *ozaenae* showed good tolerance to 10% concentration while *Pseudomonas* sp. showed good tolerance to 20% concentrations. *Shigella sonnei*, and

*Escherichia coli* showed good tolerance to 10% concentration of crude oil and 1% concentration of diesel fuel. *Proteus mirabilis* showed good tolerance to 1% concentration of crude oil and diesel fuel. However, for Gram positive organisms isolated, only *Bacillus* sp. showed good tolerance to 20% concentrations of crude oil and diesel fuel. *Micrococcus luteus* showed good tolerance to 10% concentration of crude oil but 5% concentration of diesel fuel, *Staphylococcus saprophyticus* showed good tolerance to 5% concentration of crude oil but 1% concentration of diesel fuel. Crude oil and diesel fuel were extremely toxic even at very low concentration of 1% to *Lactobacillus delbrueckii*.

## Conclusion

From this study, it has been established that crude oil and diesel fuel were toxic to bacteria isolated from pristine soil. Diesel fuel being a refinery product was more toxic to bacterial isolates because it contains light fraction of hydrocarbons which is more soluble and bioavailable. The toxicity of crude oil and diesel fuel increased with increase in concentration. The predominant crude oil and diesel oil tolerant bacterial species were belonged to the family Enterobacteriaceae, including *Erwinia* sp. and *Klebsiella* sp. These organisms, *Erwinia cacticida* and *Klebsiella pneumoniae* subsp. *ozaenae* could play a major role in the bioremediation of oil contaminated environment.

## Conflict of interest statement

Authors declare that they have no conflict of interest.

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